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## (54) Beta-d-glucan, and its production and uses.

(57) A novel beta-D-glucan derived from a microorganism of the genus Aureobasidium. The beta-D-glucan exhibits physiological activities such as acceleration of the excretion of heavy metals, anticholesteremic-activity, and an antioncotic-activity via the cellular immune system.

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# BETA-D-GLUCAN, AND ITS PRODUCTION AND USES

The present invention relates to a novel beta-D-glucan, and its production and uses, for example, in chemicals, foods and pharmaceuticals.

In this specification, parts and percentages are expressed by weight based on the dry solid, unless specified otherwise, and "Glc" represents a beta-linked D-glucopyranose residue.

Certain beta-D-glucans have drawn attention as pharmaceuticals or its material because the glucans show physiological activity such as blood-sugar lowering- and anti-cholesteremic-activity, and an antioncotic-activity via the cellular immune system.

Particularly, those beta-D-glucans that exhibit an antioncotic-activity to malignant tumors have drawn attention as antioncotics: for example, pachyman reported by H. Saito et al., Agricultural Biological Chemistry, Vol. 32, pp. 1261-1269 (1968); lentinan reported by T. Sasaki and N. Takasuka, Carbohydrate Research, Vol. 47, pp. 99-104 (1976); schizophyllan reported by K. Tabata et al., Carbohydrate Research, Vol. 89, pp. 121-135 (1981); and the beta-D-glucan reported by A. Misaki et al., Carbohydrate Research, Vol. 92, pp. 115-129 (1981).

These beta-D-glucans are prepared from the fruiting body of a microorganism of the class Basidiomycetes, and they require a long prepreation time and result in an insufficient yield.

The present inventors have studied beta-D-glucans which can be extensively used, for example, in pharmaceuticals, foods and chemicals.

As the result, the present inventors found in a culture of the genus <u>Aureobasidium</u> a novel beta-D-glucan having the following physicochemical properties:

(a) Elemental analysis;

C≒44.1%   H≒6.18%

N<0.1%   Ash<0.01%

(b) Molecular weight (by gel filtration);

100,000 to 500,000 daltons

(c) Melting point;

decomposable at about 230°C

(d) Specific rotation $[\alpha]_D^{25}$;

±4 degrees

($\ell$=1, c=1.0%, 1 N NaOH)

(e) Infrared spectrum (by the KBr tablet method);

as illustrated in FIG.1

(f) Solubility;

readily soluble in both 0.5 N NaOH and dimethyl sulfoxide; soluble in water; and insoluble in methanol, ethanol, acetone and chloroform

(g) Color reaction;

anthrone-sulfuric acid reaction   positive

phenol-sulfuric acid reaction   positive

| | |
|---|---|
| carbazole reaction | negative |
| ninhydrin reaction | negative |
| iodine reaction | negative |

(h) Properties in aqueous solution;

0.1% aqueous solution of a lyophilized preparation is neutral or slightly acidic

(i) Appearance;

white powder

More particularly, the present inventors found that methylation analysis of the beta-D-glucan gave molecular ratios of 11 to 15 for 2,4,6-tri-O-methyl-D-glucose, 0.8 to 1.2 for

2,3,4-tri-O-methyl-D-glucose, 0.6 to 1.0 for 2,3,6-tri-O-methyl-D-glucose, and 0.8 to 1.2 for 2,4-di-O-methyl-D-glucose against 1.0 mole of 2,3,4,6-tetra-O-methyl-D-glucose; or, 3 to 5 for 2,4,6-tri-O-methyl-D-glucose, 0.3 to 0.5 for 2,3,4-tri-O-methyl-D-glucose, 0.2 to 0.4 for 2,3,6-tri-O-methyl-D-glucose, and 0.9 to 1.2 for 2,4-di-O-methyl-D-glucose against 1.0 mole of 2,3,4,6-tetra-O-methyl-D-glucose.

These findings confirmed that the beta-D-glucan was totally different from the polysaccharide derived from Aureobasidium pullulans as reported in R. G. Brown and B. Lindberg, Acta Chemica Scandinavica, Vol.21, pp.2383-2389 (1967). The present inventors named this novel beta-D-glucan as "Aureobasillan".

The following further confirmed this:

(a) Elemental analysis;

| | | |
|---|---|---|
| observed | C≒44.1% | H≒6.18% |
| | N<0.1% | Ash<0.01% |
| calculated | C=44.4% | H=6.17% |

(b) Molecular weight (by gel filtration);
100,000 to 500,000 daltons

(c) Melting point;
decomposable at about 230°C

(d) Specific rotation $[\alpha]_D^{25}$;
±4 degrees
($\ell$=1, c=1.0%, 1 N NaOH)

(e) Infrared spectrum (by the KBr tablet

method);

as illustrated in FIG.1

(f) Solubility;

readily soluble in both 0.5 N NaOH and dimethyl sulfoxide, soluble in water and insoluble in methanol, ethanol, acetone and chloroform

(g) Color reaction;

| | |
|---|---|
| anthrone-sulfuric acid reaction | positive |
| phenol-sulfuric acid reaction | positive |
| carbazole reaction | negative |
| ninhydrin reaction | negative |
| iodine reaction | negative |

(h) Properties in aqueous solution;

0.1% aqueous solution of a lyophilized preparation is neutral or slightly acidic

(i) Appearance;

white powder

(j) Sugar components;

Aureobasillan was completely hydrolyzed by subjecting it to an inorganic or organic acid, for example, in 72% sulfuric acid at ambient temperature for 5 minutes, diluting the mixture with 7 volumes of water, and incubating it at 100°C for 4 to 5 hours; or by heating it at 100°C for 6 hours in 2 M

trichloroacetic acid. The resultant saccharide was analyzed by the paper chromatography, gas chromatography or glucose-oxidase/peroxidase method, and the resultant saccharide was D-glucose.

(k) Linkage;

i) The low specific rotation $[\alpha]_D^{25}$, i.e., ±4 degrees, and the infrared absorption at about 890 $cm^{-1}$ indicate that all or most of the glucose residues constructing Aureobasillan are linked in beta-fashion.

ii) Methylation analysis of Aureobasillan was carried out as follows: Aureobasillan was methylated in dimethylsulfoxide solution in accordance with the Hakomori method using methylsulfinyl carbanion and methyl iodide. The resultant methyl derivatives were acid-hydrolyzed, and then converted into alditol acetate. The gas chromatography and gas chromatography-mass spectrometry revealed that the alditol acetate had the following molecular ratios. An Aureobasillan specimen (Aureobasillan A) which had a relatively low molecular weight and did not adsorb onto DEAE-cellulose showed molecular ratios of 11 to 15 for

2,4,6-tri-O-methyl-D-glucose; 0.8 to 1.2 for 2,3,4-tri-O-methyl-D-glucose; 0.6 to 1.0 for 2,3,6-tri-O-methyl-D-glucose; and 0.8 to 1.2 for 2,4-di-O-methyl-D-glucose, against 1 mole of 2,3,4,6-tetra-O-methyl-D-glucose. The other Aureobasillan specimen (Aureobasillan B) which had a relatively high molecular weight and did not adsorb onto DEAE-cellulose showed molecular ratios of 3 to 5 for 2,4,6-tri-O-methyl-D-glucose; 0.3 to 0.5 for 2,3,4-tri-O-methyl-D-glucose; 0.2 to 0.4 for 2,3,6-tri-O-methyl-D-glucose; and 0.9 to 1.2 for 2,4-di-O-methyl-D-glucose, against 1.0 mole of 2,3,4,6-tetra-O-methyl-D-glucose.

(iii) A water-insoluble polymer fraction obtained by the moderate Smith degradation of Aureobasillan was methylated and acid-hydrolyzed to obtain a mixture of relatively large amount of 2,4,6-tri-O-methyl-D-glucose and a small amount of 2,3,4,6-tetra-O-methyl-D-glucose. The mixture was subjected to the action of an endo-type beta-1,3-glucanase to obtain a product which contained mainly, laminaribiose, and small amounts of glucose and $^{2}$6-O-beta-

glucosyllaminaribiose. These facts showed that a substantial part of the beta-1,6 linkages was incorporated, adjacently to a beta-1,3 linkage, in the main chain which was repeatedly linked in beta-1,3 fashion.

These facts revealed that Aureobasillan was entirely different from conventional beta-D-glucans, based on the facts that it had beta-1,6 linkages at a constant ratio in the main chain which was repeated in beta-1,3 linkages and had short side chains which were branched at a constant ratio at the C-6 positions of the glucose residues; and that the side chains contained a substantial amount of beta-1,4 linkages in addition to beta-1,6 linkages.

Thus, Aureobasillan includes Aureobasillan A which is composed of repeating units as represented by the following formulae:

formula I

$$\left[ \left[ \rightarrow 3)\mathrm{Glc}(1 \rightarrow \right]_X 3)\mathrm{Glc}(1 \rightarrow \left[ 3)\overset{\begin{array}{c}\mathrm{Glc}(1 \rightarrow 6)\mathrm{Glc}\\ 1\\ \downarrow\\ 6\end{array}}{\mathrm{Glc}}(1 \rightarrow \right]_Y \right]_m$$

and formula II

$$\left[\begin{array}{l}\qquad\qquad\quad \mathrm{Glc}(1\rightarrow 4)\mathrm{Glc} \\ \qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\quad 6 \\ \left[\rightarrow 3)\mathrm{Glc}(1\right]_{X'}\rightarrow 3)\mathrm{Glc}(1\left[\rightarrow 3)\mathrm{Glc}(1\right]_{Y'}\rightarrow 6)\mathrm{Glc}(1\end{array}\right]_{n}\rightarrow$$

wherein each of X, Y, X' and Y' independently represents 1 or an integer greater than 1, each of X+Y and X'+Y' independently represents an integer in the range of from 11 to 15, and the ratio m:n is from 1:3 to 1:5, and the Aureobasillan also includes Aureobasillan B which is mainly composed of repeating units as represented by the following formulae:

formula I

$$\left[\begin{array}{l}\qquad\qquad\qquad\qquad \mathrm{Glc}(1\rightarrow 6)\mathrm{Glc} \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad 6 \\ \left[\rightarrow 3)\mathrm{Glc}(1\right]_{X}\rightarrow 3)\mathrm{Glc}(1\left[\rightarrow 3)\mathrm{Glc}(1\right]_{Y}\end{array}\right]_{m}\rightarrow$$

and formula II

$$\left[\begin{array}{l} \qquad \mathrm{Glc}(1\rightarrow 4)\mathrm{Glc} \\ \qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\quad 6 \\ \left[\rightarrow 3)\mathrm{Glc}(1\right]_{X'}\rightarrow 3)\mathrm{Glc}(1\left[\rightarrow 3)\mathrm{Glc}(1\right]_{Y'}\rightarrow 6)\mathrm{Glc}(1\rightarrow \end{array}\right]_n$$

wherein each of X, Y, X' and Y' independently represents 1 or an integer greater than 1, each of X+Y and X'+Y' independently represents an integer in the range of from 3 to 5, and the ratio m:n is from 1:3 to 1:5.

To prepare Aureobasillan, a seed culture of, for example, Aureobasidium pullulans IFO 4464, IFO 4875, IFO 6353, IFO 6401, IFO 6725 or Aureobasidium mansoni IFO 9233 is inoculated to a solid culture medium or liquid culture medium, containing appropriate nutrients such as a carbon source, nitrogen source and minerals, and the mixture was subjected to a static culture or submerged culture or shaking culture to accumulate Aureobasillan in the culture, followed by recovery of the accumulation.

Pullulan can be favorably accumulated in the culture along with Aureobasillan.

Any substance can be used as the nutrient in the culture medium so long as Aureobasillan is produced in the

culture medium: for example, glycerol, xylose, glucose, sorbitol, fructose, maltose, isomaltose, maltitol, sucrose, lactose, cellobiose, maltotriose, maltotetraose, partial starch hydrolysates having a DE (dextrose equivalent) of 10 to 70, and exhausted molasses can be favorably used as the carbon source. To produce Aureobasillan and pullulan simultaneously, cultivation is favorably carried out with a liquid culture medium containing about 3 to 20 w/v % of one or more of the above-mentioned saccharides under aerobic conditions. Synthetic compounds, such as, nitrates, ammonium salts and urea; and natural organic compounds, such as, polypeptones, yeast extracts, malt extracts, corn steep liquor, extracts of defatted soybean, peptides, and amino acids can be used as the nitrogen source.

Mineral(s) may be selected from one or more of phosphate, potassium salt, sulphate, magnesium salt, and, if necessary, ferrate, manganate, and calciums salts.

The pH and temperature during cultivation are those under which the microorganism grows and produces Aureobasillan: generally, pH 2.0 to 9.0 and a temperature of 15 to 35°C. The cultivation is continued until Aureobasillan production maximizes: generally 1 to 10 days in the case of using a liquid culture medium under aeration-agitation conditions.

Aureobasillan can be recovered from the culture, for example, by collecting the cells from the culture, and separating the Aureobasillan from the cells. Pullulan can be recovered by conventional procedure from the cell-free filtrate or supernatant of

the culture.

Aureobasillan can be recovered from the cells by allowing cell-walls derived from cells or cell debris to contact with an eluent, for example, hot water, dilute acid or dilute alkali, particularly, an aqueous alkaline solution of a pH exceeding 7.0, more particularly, 0.01 to 4.0 N dilute aqueous solution of potassium hydroxide, sodium hydroxide, magnesium hydroxide or calcium hydroxide, to elute the Aureobasillan, followed by recovery of the Aureobasillan solution.

The Aureobasillan solution is, if necessary, after concentration, neutralization, decoloration, deionization and purification with an activated carbon and ion-exchange resins in the usual manner, concentrated and dehydrated to obtain a white Aureobasillan powder.

An ultracentrifugally- and electrophoretically-homogeneous high-purity Aureobasillan fraction can be easily obtained by treating an aqueous Aureobasillan solution with an organic precipitant such as methanol, ethanol, isopropanol and acetone, or by chromatographying the aqueous solution.

The resultant high-purity Aureobasillan can be easily dehydrated and pulverized. To effect such dehydration, conventional methods such as flow drying, hot air drying, spray drying, drum drying and lyophilizing can be chosen.

In order to prepare a polyol-type Aureobasillan from the Aureobasillan thus obtained, one part of the latter Aureobasillan is dissolved or suspended in about 50 to 500 parts

aqueous solution containing about 0.01 to 0.5 M of periodic acid or periodate such as meta-sodium periodate and meta-potassium periodate, and allowed to react, generally, at a pH in the range of from 3 to 8. This operation is generally carried out under mild conditions, for example, at ambient temperature in the dark, particularly, at a temperature of 15°C or lower in the dark for 1 to 5 days, to complete the oxidation reaction. The polyaldehyde-type Aureobasillan thus obtained wherein the side chains have been mainly oxidized can be advantageously used as a carrier for an immobilized enzyme by the covalent method because of the high reactivity.

In order to reduce the polyaldehyde-type Aureobasillan, the residual periodic acid in the reaction mixture is consumed by the addition of ethylene glycol, or removed by dialysis, thereafter the resultant product is reduced with a reducing agent. If desired, polyaldehyde-type Aureobasillan may be recovered from the reaction mixture, prior to the reduction reaction.

Any reduction method can be employed as long as the oxidized Aureobasillan is reduced. For example, hydrogenation using a nickel catalyst or reduction using sodium borohydride may be used. The nickel catalyst in the reaction mixture is removed or the sodium borohydride in the reaction mixture is decomposed by the addition of an organic acid in the usual manner. Then the reaction mixture is subjected to repeated precipitations using an organic precipitant, if necessary, decolored, deionized and purified with activated charcoal and ion-exchange resins, and concentrated to obtain a polyol-type

Aureobasillan syrup which is easily preparable into powder. In the polyol-type Aureobasillan, the beta-1,3 linked glucose residues are left unchanged, but the remaining beta-1,6 linked glucose residues in the side chains are ring cleaved.

The intact and polyol-type Aureobasillans thus obtained can be freely used, for example, in chemicals, foods and pharmaceuticals. In the case of chemicals, intact Aureobasillan can be freely prepared alone or in combination with one or more materials into a composition or shaped body, for example, granules, tablets and sheets, because intact Aureobasillan is a water soluble saccharide. Polyol-type Aureobasillan is suitable for use as a sizing agent, viscosity-imparting agent, emulsifier, fiber, film and coating membrane, because polyol-type Aureobasillan is a readily water soluble poly-saccharide. In the case of foods, intact and polyol-type Aureobasillans are suitable for use in health promoting foods, because they are edible, non-toxic, tasteless, non- or hardly-assimilable fibers having an anticholesteremic-activity as well as an activity of accelerating excretion of heavy metals.

Intact and polyol-type Aureobasillans can be freely used in pharmaceuticals, particularly in antioncotics because they activate the cellular immune system to exhibit a high antioncotic-activity. Thus, intact and polyol-type Aureobasillans can be used alone or in combination with one or more agents and/or adjuvants, for example, in the form of injections or internal- or external-medicines in the therapy of malignant tumors which are sensitive to intact or polyol-type Aureobasillan, for example, breast cancers, lung cancers, bladder cancers, hysterocarcinomas, colorectal cancers, gastric cancers, leukemia, lymphoma and skin cancer.

The efficacy of such therapy may be augmented by a combined use with one or more other antioncotics, for example, alkylating agents such as cyclophosphamide and nimustine hydrochloride; antimethabolites such as methotrexate, fluorouracil and tegafur; antibiotics such as bleomycin, mytomycin C and actinomycin C; alkaloids such as vincristine sulfate and vinblastine sulfate; hormones such as prednisone, methyltestosterone and conjugated estrogen; and lymphokines such as interferon, lymphotoxin, tumor necrosis factor and interleukin 2.

The present invention will now be illustrated further, by way of example only, by the following experiments which describe in more detail the antioncotic-activity, toxicity, efficacy and dosage of intact and polyol-type Aureobasillans.

Experiment 1

Ten 4 week-old ICR-JCL female mice were implanted with about $6 \times 10^6$ cells of Sarcoma-180 tumor line into their right groins. From the first day following the implantation, 0.1 ml saline containing 1, 5 or 10 mg/kg mouse of either Aureobasillan, obtained by the method described in Example 1, or a polyol-type Aureobasillan, obtained by the method described in Example 3, was daily injected intraperitoneally into the mice at 1 dose per day over a period of the following 10 days. As a control, saline solution free of the intact or polyol-type Aureobasillan was administered in the same manner. On the thirty-fifth day after the implantation, the tumors, formed in the animals, were extracted and weighed. The tumor inhibitory ratio (%)

was determined by comparing the weight of the tumor of the group which was administered with the intact or polyol-type Aureobasillan with that of the control group.

$$\text{Tumor inhibitory ratio (\%)} = \frac{(A-B)}{A} \times 100$$

where A is the average tumor weight of the 10 control mice; and B, the average of the 10 mice which had been administered with the intact or polyol-type Aureobasillan.

The results were as shown in Table 1.

Table 1

| | | Dose mg/kg/day×time | Average tumor weight (g) | Tumor inhibitory ratio (%) | Number of mice completely involuted | Remarks |
|---|---|---|---|---|---|---|
| | | 0 × 10 × 1 | 9.6 ± 1.4 | - | 0 | control |
| Intact Aureobasillan | A | 1 × 10 × 1 | 0.3 | 96.9 | 9 | present invention |
| | | 5 × 10 × 1 | 0 | 100 | 10 | present invention |
| | | 10 × 10 × 1 | 0 | 100 | 10 | present invention |
| | B | 1 × 10 × 1 | 0.4 | 95.8 | 9 | present invention |
| | | 5 × 10 × 1 | 0 | 100 | 10 | present invention |
| | | 10 × 10 × 1 | 0 | 100 | 10 | present invention |
| Polyol-type Aureobasillan | A | 1 × 10 × 1 | 0 | 100 | 10 | present invention |
| | | 5 × 10 × 1 | 0 | 100 | 10 | present invention |
| | | 10 × 10 × 1 | 0 | 100 | 10 | present invention |
| | B | 1 × 10 × 1 | 0 | 100 | 10 | present invention |
| | | 5 × 10 × 1 | 0 | 100 | 10 | present invention |
| | | 10 × 10 × 1 | 0 | 100 | 10 | present invention |

These results revealed that intact and polyol-type Aureobasillans very effectively inhibited the growth of malignant tumors.

The data from this experiment have been deemed applicable to other warm-blooded animals, for example, mammals such as humans, cows, horses, dogs, cats, rabbits and rats, and birds such as chickens and pigeons.

Experiment 2

Ten $BDF_1$ male mice, about 25 g each, were implanted on their dorsal area with a 2 mm-square piece of Lewis lung cancer tissue. On the eighth day after the implantation, 0.1 ml saline containing 0.02, 0.1 or 1 mg/kg mouse of either intact or polyol-type Aureobasillan, obtained by the method described in Example 1 or 3, was daily injected intraperitoneally into the mice at 2 doses per day over a period of the following 10 days. As a control, saline solution free of intact or polyol-type Aureobasillan was administered in the same manner. On the twenty-third day after the implantation, the tumors were weighed. The tumor inhibitory ratio (%) was determined in the same manner as in Experiment 1.

The results were as shown in Table 2.

Table 2

| | | Dose mg/kg/day×time | Average tumor weight (g) | Tumor inhibitory ratio (%) | Remarks |
|---|---|---|---|---|---|
| | | 0 × 10 × 2 | 8.4 ± 0.5 | - | control |
| Intact Aureobasillan | A | 0.02 × 10 × 2 | 5.7 ± 0.4 | 32.1 | present invention |
| | | 0.1 × 10 × 2 | 5.0 ± 0.6 | 40.5 | present invention |
| | | 1 × 10 × 2 | 3.9 ± 0.5 | 53.6 | present invention |
| | B | 0.02 × 10 × 2 | 5.9 ± 0.5 | 29.8 | present invention |
| | | 0.1 × 10 × 2 | 5.1 ± 0.4 | 39.3 | present invention |
| | | 1 × 10 × 2 | 4.2 ± 0.6 | 50.0 | present invention |
| Polyol-type Aureobasillan | A | 0.02 × 10 × 2 | 5.3 ± 0.6 | 36.9 | present invention |
| | | 0.1 × 10 × 2 | 4.0 ± 0.7 | 52.4 | present invention |
| | | 1 × 10 × 2 | 2.9 ± 0.5 | 65.5 | present invention |
| | B | 0.02 × 10 × 2 | 5.4 ± 0.6 | 35.7 | present invention |
| | | 0.1 × 10 × 2 | 4.2 ± 0.7 | 50.0 | present invention |
| | | 1 × 10 × 2 | 3.0 ± 0.6 | 64.3 | present invention |

These results revealed that intact and polyol-type Aureobasillans were very efficacious against malignant tumors, such as lung cancer, the treatment of which has been deemed very difficult.

Experiment 3

Both intact and polyol-type Aureobasillans, obtained by the method described in Experiment 1 or 3, were tested for acute toxicity by administering either of them orally, intraperitoneally or intraveneously into 4 week-old mice by conventional methods.

The results obtained showed that both beta-glucans were extremely low in toxicity, and their highest possible dosage resulted in no death of the mice.

The $LD_{50}$ (median lethal dose) of both beta-D-glucans were, not necessarily correct, 20 g/kg or higher when orally administered; 5 g/kg or higher when intraperitoneally administered; and 1.5 g/kg or higher when intraveneously administered.

As is evident from these Experiments, intact and polyol-type Aureobasillans would be safe in view of their effective doses. Thus, intact and polyol-type Aureobasillans are suitable for the therapeutic treatment of malignant tumors. Any administration method can be employed as long as it is effective for the treatment of malignant tumors; for example, subcutaneous-, intramuscular- or intravenous-injection; oral administration; suppository administration; external application; and instillation.

The daily dose of intact and polyol-type Aureobasillans for adults is generally 0.1 mg to 500 g, in particular, 10 mg to 500 g for oral administration, and 0.1 mg to 100 g for injection, but varies with the administration method employed.

The following Examples further illustrate the present invention.

Example 1

Aureobasillan

A seed culture of *Aureobasidium pullulans* IFO 4464 was inoculated in a 20 liters culture medium conprising 10% of partial starch hydrolysate (D.E.40), 0.2% of $K_2HPO_4$, 0.2% of peptone, 0.2% of NaCl, 0.04% of $MgSO_4$ heptahydrate and 0.001% of $FeSO_4$ heptahydrate, and the mixture was cultivated at 27°C for 5 days under aeration-agitation conditions. The cells were separated with a precoated filter.

The resultant filtrate was purified, concentrated, and pulverized in conventional manner to obtain about 1.4 kg of a pullulan powder.

The separated cells, about 200 g as dry solid, were washed with hot water, fed to a "Dino Mill" cell crusher manufacturered by Willy A. Bachofen, Basel, Switzerland, and centrifuged to collect the cell debris which was then defatted with acetone and added with 4 liters of 0.5 N sodium hydroxide. The mixture was then gently stirred in a nitrogen atmosphere at 25°C for 4 hours, followed by centrifugation. The resultant seupernatant was dialyzed against water, dehydrated and concentrated to obtain about 8 g of a crude Aureobasillan. One g of the Aureobasillan was dissolved in 200 ml of 0.01 M phosphate buffer (pH 7.8), and the resultant solution was applied on a column of DEAE-cellulose. The non-adsorbed fraction was then dialyzed, concentrated, lyophilized and pulverized to obtain about 400 mg of a white Aureobasillan A powder.

The molecular weight of the product was 100,000 to 200,000 daltons as determined by gel chromatography using a column of Sepharose CL-6B, registered trade mark of Pharmacia, Uppsala, Sweden.

The specific rotation $[\alpha]_D^{25}$ of a 1.0 % aqueous solution, prepared by dissolving the powder with 1 N sodium hydroxide in a nitrogen atmosphere, was plus one degree.

The infrared spectrum of the powder determined by the KBr tablet method was as shown in FIG. 1.

The fraction which had adsorbed onto the column of DEAE-cellulose was eluted with 0.1 N aqueous solution of sodium hydroxide, purified similarly as in the non-adsorbed fraction, and pulverized to obtain about 500 mg of an Aureobasillan B powder.

The molecular weight and specific rotation $[\alpha]_D^{25}$ of the Aureobasillan B powder, as determined in a manner similar to that described for Aureobasillan A, were 350,000 to 450,000 daltons and minus one degree respectively. The infrared spectrum of the Aureobasillan B powder was approximately the same absorption pattern as that shown in FIG. 1.

The Aureobasillans thus obtained can be suitably used in chemicals, foods and pharmaceuticals.

Example 2

Twenty liters of a liquid culture medium, consisting of 8 w/v % of sucrose, 0.2 w/v % of yeast extract, 0.3 w/v % of corn steep liquor, 0.1 w/v % of $NH_4NO_3$, 0.1 w/v % of $K_2HPO_4$, 0.05 w/v % of $MgSO_4$

heptahydrate, 0.05 w/v % of KCl, 0.0001 w/v % of $MnSO_4$ tetrahydrate and water, was sterilized at 120°C for 20 minutes, cooled, adjusted to pH 7.0, and inoculated with a seed culture of Aureobasidium pullulans IFO 6353. The mixture was then cultivated at 30°C for 4 days under aeration-agitation conditions. The resultant culture was treated in a manner similar to that described in Example 1 to recover from the filtrate of the culture about 0.7 kg of a pullulan powder and, from the cells, about 7 g of a crude Aureobasillan.

One gram of the crude Aureobasillan was dissolved in 500 ml of 0.01 N aqueous sodium hydroxide solution, decolorized with an activated carbon, deionized and purified with ion-exchange resins (H- and OH-forms) in conventional manner, concentrated, lyophilized and pulverized to obtain about 800 mg of a high-purity white Aureobasillan powder. The specific rotation $[\alpha]_D^{25}$ of the powder containing Aureobasillans A and B was zero degree.

Similar to the preparation obtained by the method in Example 1, the powder can be extensively used.

Example 3

Polyol-type Aureobasillan

Ten grams of either Aureobasillan A or B, obtained by the method of Example 1, was suspended in 500 ml of an aqueous solution containing 6.6 g of meta-sodium periodate, and the mixture was then stirred at 10°C in the dark for 7 days to effect oxidation. The reaction mixture was dialyzed against water. The liquid inside the dialyzing means was added with 1.5 g of sodium borohydride, and the

mixture was allowed to effect reduction at ambient temperature for 2 days, and adjusted to pH 6.0 with acetic acid to decompose the remaining sodium borohydride. Thereafter, the resultant was dialyzed against water.

The liquid inside the dialyzing means was added with 3 volumes of methanol, and the resultant precipitate was centrifuged, dissolved in water, precipitated, dissolved in water, lyophilized and pulverized to obtain about 7.5 g of a pulverulent polyol-type Aureobasillan A or B.

The products are superior in water-solubility to intact Aureobasillan, and are suitable for use in chemicals, foods and pharmaceuticals.

Example 4

Film

A 10 w/v % aqueous solution of a polyol-type Aureobasillan A, obtained by the method of Example 3, also containing glycerine in an amount of 10% against the dry solid of the polyol-type Aureobasillan A, was cast on a glass plate, and dehydrated with 70°C air to obtain a film having a satisfactory transparency, brightness, and physical strength. Since the product exhibits a high oxygen-impermeability, it can be used to coat or pack products which are susceptive to oxidation to prolong their storage period and shelf lives.

Example 5

Fiber

A 20 w/v % aqueous solution of a polyol-type Aureobasillan B, obtained by the method of Example 3, was heated to 80°C, and extruded into fiber in air at ambient temperature through a cylindrical nozzle 0.3 mm in diameter and 1 mm in length, pressure of 3 $kg/cm^2$, and the fiber was wound around a core.

The obtained fiber, thickness, about 20 u, was satisfactory in physcial strength. The fiber can be twisted, knitted or woven. The fiber is hydrophilic, non-toxic, and non-skin-irritative, so that the fiber can be used, for example, as absorbent cotton, sanitary cotton, gauze, gut in therapy, or shaped body in therapy of malignant tumors. By utilizing the satisfactory moisture-absorbing ability, non-electrifiability, and stainability, the product can be used in blended fiber for clothes such as underwear.

Example 6

Coating membrane

Fresh eggs, soaked in a 35°C aqueous solution containing 0.5 w/v % of a crude Aureobasillan, obtained by the method of Example 2, for 30 seconds within 10 hours after the eggs had been laid, and dehydrated in a 30°C air for 1 hour to form a coating membrane over the surface of the eggs, were stored at ambient temperature, i.e., 15 to 25°C, and their shelf lives were compared with the shelf life of uncoated control eggs. The results revealed that the coating kept the eggs fresh about 5 to 10 times longer than the uncoated control eggs.

Example 7

Cup

An Aureobasillan A powder, obtained by the method of Example 1, was sprinkled with water to attain a moisture content of about 30% with mixing, and fed to an extruder to form a rod which was then cut into pellets, 25 mm in diameter, 4 mm in length. The pellets were then fed to an injection moulder, and injected at a resin temperature of 120°C to obtain cups having a satisfactory physical strength and transparency.

Example 8

Fertilizer rod

Seventy parts of a compound fertilizer (N=14%, $P_2O_5$=8%, $K_2O$=12%), 10 parts of a crude Aureobasillan, obtained by the method of Example 1, 15 parts of calcium sulfate and 5 parts of water were sufficiently mixed, and heated to 80°C in an extruder (L/D=20, compression ratio=18 and dice diameter=30 mm) to obtain a fertilzier rod.

The product is convenient to carry and does not necessarily require a container. The product has a physical strength sufficient for total layer application, and the releasing ratio can be controlled by varying the formulation of the components in the compound fertilizer.

Example 9

Capsule

An aqueous solution of 5 w/v % polyol-type Aureobasillan A,

obtained by the method of Example 3, and 10 w/v % of gelatin was heated to 60°C, and degassed. The end of a metal rod was soaked in the solution, immediately pulled out, and dehydrated gradually in air at 40°C to obtain a high-quality hard capsule having a satisfactory elasticity, transparency and brightness. The product can be favorably used for packaging, for example, a suppository and a medicament for oral administration.

Example 10

Adhesive

A mixture of 30 parts of dimethylsulfoxide, 25 parts of water, 2 parts of a high-purity Aureobasillan, obtained by the method of Example 2, 8 parts of pullulan, and 2 parts of dibenzylidene xylitol was dissolved by 1 hour-stirring at 90°C, injected into a cylindrical lipstick-type container, 14 mm in diameter, 50 mm in height, equipped with an up and down moving mechanism, and cooled at ambient temperature to obtain a solid-type adhesive. The adhesive can be applied on a kraft to form a layer of uniform thickness on paper, and the adhesive also exhibits a sufficient initial adhesion.

Example 11

Alimentary pastes

Seventy parts of rice powder, 20 parts of potato starch, 10 parts of wheat flour, 2 parts of an Aureobasillan A, obtained by the method of Example 1, and 40 parts of 10% aqueous saline solution were homogeneously mixed, steamed, sufficiently kneaded, and prepared into a dough, followed by overnight standing. The resultant dough was cut

into alimentary pastes, and placed in boiling water for 3 minutes. The alimentary pastes had a sufficient "koshi (softness and chewiness)".

Example 12

"Chinmi (a type of relish)"

A "soboro (a minced meat)", prepared by roasting in a frying pan 30 parts of minced chicken, 2 parts of sucrose, 2 parts of soy sauce and 6 parts of "mirin (sweet sake)", was added with 3 parts of an Aureobasillan B powder, obtained by the method of Example 1, sufficiently kneaded, pressed at about 50 kg/cm$^2$ while heating at about 150 to 170°C to obtain about 1 cm thick sheets. The sheets were cut into pieces to obtain the captioned product having a delicate flavour. The product was suitable as a relish or snack for children.

Example 13

Fish paste product

Four thousand parts of a thawed walleye pollack paste was homogeneously ground and kneaded with 80 parts of maltose, 80 parts of sodium glutaminate, 200 parts of potato starch, 300 parts of ice water, 12 parts of sodium tripolyphosphate, 120 parts of salt, and 100 parts of an aqueous solution obtaining 10 parts of a polyol-type Aureobasillan A, obtained by the method of Example 3, and 1 part of sorbitol. About 120 g aliquot bars were steamed in such manner that the products were heated up to about 80°C over a period of 30 minutes. Then, the products were cooled at ambient temperature, and allowed to stand at 4°C for 24 hours to obtain fish paste products.

These products had satisfactory gloss and biting properties.

Example 14

"Koromo (batter)" for fried food

One part of a high-purity Aureobasillan, obtained by the method of Example 2, was added to 100 parts of weak flour, and the resultant mixture was admixed with 300 parts of water to obtain "koromo". "Tane (seafoods and vegetables for fried food)" such as lobster or sweet potato, was coated with the "koromo", and fried. The fried "koromo" had desirable biting properties and adhesion to "Tane".

Example 15

Ice cream

Seventy parts of 40% cream, 200 parts of sweetened condensed milk, 460 parts of whole milk, 20 parts of skim milk, 5 parts of sucrose, 5 parts of maltose and 4 parts of 1.0 w/v % aqueous solution of a high-purity Aureobasillan, obtained by the method of Example 2, were mixed by heating and pasteurized at 70°C for 30 minutes, cooled quickly to 3 to 4°C with a homogenizer, aged overnight, and fed to a freezer.

The product was a tasty ice cream.

Example 16

Lemon jelly

Three parts of agar and 5 parts of a polyol-type Aureobasillan B, obtained by the method of Example 3, were added with 200 parts of water and 50 parts of sucrose, and the mixture was dissolved by heating, and cooled to 65°C.

The mixture was then added with 350 parts of carbonated water containing small amounts of flavors including lemon flavor, and the resultant was poured into cups, and cooled to obtain a lemon jelly having a satisfactory gloss. The product is a health promoting food containing polyol-type Aureobasillan B which is an edible fiber.

Example 17

Yoghurt

One hundred and seventy-five parts of a skim milk powder, 80 parts of sucrose, 50 parts of maltose, and 30 parts of an Aureobasillan A, obtained by the method of Example 1, were dissolved in 1,200 parts of water while stirring, and the mixture was fed to a homogenizer, pasteurized at about 85°C for 30 minutes, and cooled to 40°C.

The mixture was added with 30 parts of a starter prepared with lactic acid bacteria in commercialized yoghurt, and the resultant was cultured at 37°C for 8 hours to obtain a yoghurt-gel.

The product was a tasty yoghurt having a smoothness and satisfactory gloss. The product is a health promoting food where Aureobasillan A provides an anticholesteremic-activity.

Example 18

Tablet

One hundred parts of an aqueous solution of 20 w/v % polyol-type Aureobasillan A, obtained by the method of Example 3, was admixed with 140 parts of maltose, and 20 parts of vitamin A palmitate, and the mixture was sufficiently mixed, poured over a glass plate, and air-dried. Then, the resultant product was pulverized and

fed to a tabletting machine to prepare tablets according to a conventional method. The tablets contained 100,000 IU vitamin A palmitate per gram, and, after 3-month standing at 30°C, no decrease in activity was noted. The product can be favorably used as an antioncotic which is orally administered for malignant tumors, for example, gastric cancer, duodenal cancer and rectal cancer.

Example 19

Tablet

Fourteen parts of an Aureobasillan B, obtained by the method of Example 1, and 4 parts of corn starch were sufficiently mixed with 50 parts of salicylic acid, and the resultant mixture was tabletted in a tabletting machine by a conventional method.

The product was non-hygroscopic, and sufficient, in physical strength, as well as exhibiting a satisfactory disintegration in water.

Example 20

Injection

An aqueous solution of 0.2 w/v % Aureobasillan A, obtained by the method of Example 1, was decolored with an activated charcoal, deionized and purified with ion-exchange resins (H- and OH-forms), concentrated and sterilized with a membrane filter. The filtrate was distributed into sterilized 20 ml glass-vials containing 200 mg Aureobasillan A, and lyophilized to obtain an injection. The product can be, after dissolving or suspending the Aureobasillan A in saline, favorably injected subcutaneously or intramuscularly for the

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treatment of malignant tumors, for example, breast cancer, lung cancer, hepatoma and leukemia.

Example 21

Injection

An aqueous solution of about 2 w/v % polyol-type Aureobasillan B, obtained by the method of Example 3, was decolored with an activated charcoal, deionized and purified with ion-exchange resins, concentrated and sterilized by filtration through a membrane filter as in Example 20. The filtrate was prepared into 2 w/v % isotonic solution of polyol-type Aureobasillan B, which was then distributed into 20 ml vials to obtain an injection.

The product can be favorably injected intraperitoneally or intravenously for treating malignant tumors, for example, breast cancer, bladder cancer, hysterocarcinoma, colorectal cancer and gastric cancer.

Example 22

Ointment

A high-purity Aureobasillan powder, obtained by the method of Example 2, was admixed with a small amount of liquid paraffin, and added with white petrolatum to obtain an ointment containing 10 mg Aureobasillan per gram.

The product can be favorably used for treating malignant tumors, for example, skin cancer, breast cancer and lymphoma.

As is apparent from the above, the present beta-D-glucan and its derivative, i.e., polyol-type Aureobasillan, can be favorably used

in the form of a composition in a health promoting food because they are edible, tasteless, non-toxic and non- or hardly-assimilable fibers. In addition, intact and polyol-type Aureobasillans have an anticholesteremic-activity or an activity of excreting heavy metals. Intact and polyol-type Aureobasillans can be favorably used, for example, in an antioncotic for treating malignant tumors because of their antioncotic-activity; and in a shaped body or composition, for example, in the form of tablet, film, and sheet.

Preparation of Aureobasillan on an industrial-scale can be favorably carried out by culturing a microorganism of the genus Aureobasidium and recovering the accumulated Aureobasillan from the culture. The pullulan accumulated along with Aureobasillan can also be recovered from the culture.

CLAIMS:

1. A beta-D-glucan which has the following physico-chemical properties:

(a) Elemental analysis;

$C \doteq 44.1\%$ $H \doteq 6.18\%$

$N<0.1\%$ $Ash<0.01\%$

(b) Molecular weight (by gel filtration);

100,000 to 500,000 daltons

(c) Melting point;

decomposable at about 230°C

(d) Specific rotation $[\alpha]_D^{25}$;

±4 degrees

($\ell$=1, c=1.0%, 1 N NaOH)

(e) Infrared spectrum (by the KBr tablet method);

as illustrated in FIG. 1

(f) Solubility;

readily soluble in both 0.5 N NaOH and dimethyl sulfoxide; soluble in water; and insoluble in methanol, ethanol, acetone and chloroform

(g) Color reaction;

| | |
|---|---|
| anthrone-sulfuric acid reaction | positive |
| phenol-sulfuric acid reaction | positive |
| carbazole reaction | negative |

ninhydrin reaction negative

iodine reaction negative

(h) Properties in aqueous solution;

0.1% aqueous solution of a lyophilized preparation is neutral or slightly acidic

(i) Appearance;

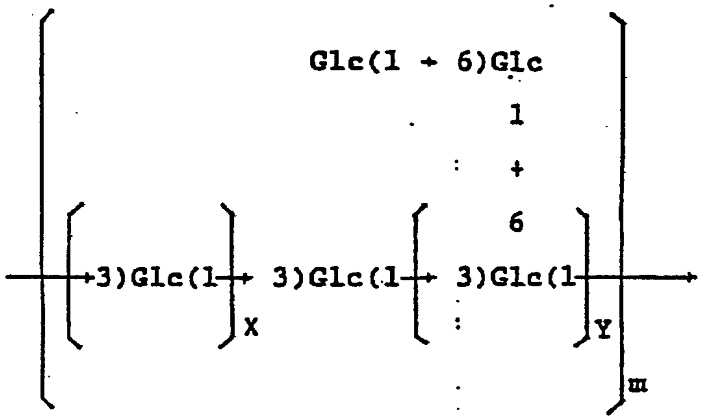
white powder

2. A beta-D-glucan according to Claim 1, which, upon methylation analysis, gives molecular ratios of 11 to 15 for 2,4,6-tri-O-methyl-D-glucose; 0.8 to 1.2 for 2,3,4-tri-O-methyl-D-glucose; 0.6 to 1.0 for 2,3,6-tri-O-methyl-D-glucose; and 0.8 to 1.2 for 2,4-di-O-methyl-D-glucose against 1.0 mole of 2,3,4,6-tetra-O-methyl-D-glucose.

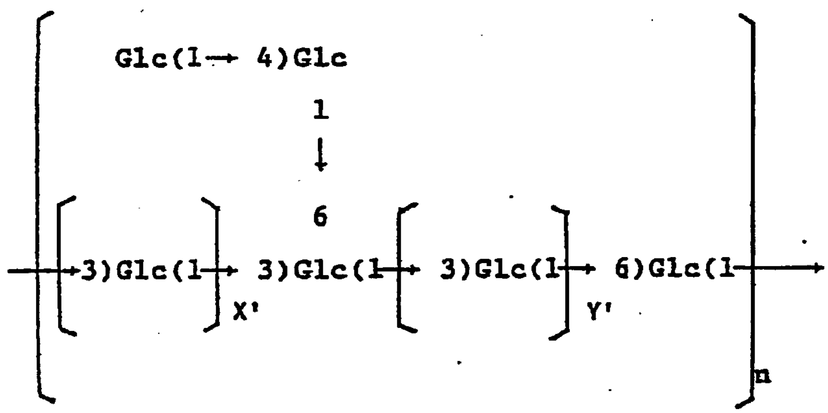
3. A beta-D-glucan according to Claim 1, which, upon methylation analysis, gives molecular ratios of 3 to 5 for 2,4,6-tri-O-methyl-D-glucose; 0.3 to 0.5 for 2,3,4-tri-O-methyl-D-glucose; 0.2 to 0.4 for 2,3,6-tri-O-methyl-D-glucose; and 0.9 to 1.2 for 2,4-di-O-methyl-D-glucose, against 1.0 mole of 2,3,4,6-tetra-O-methyl-D-glucose.

4. A beta-D-glucan according to Claim 2, which is mainly composed of repeating units as represented by the following formulae:

formula I



and formula II



wherein each of X, Y, X' and Y' independently represents 1 or an integer greater than 1, each of X+Y and X'+Y' independently represents an integer in the range of from 11 to 15, and the ratio m:n is from 1:3 to 1:5.

5. A beta-D-glucan according to Claim 3, which is mainly composed of repeating units as represented by the following formulae:

formula I

$$\left[ \left[\rightarrow 3)\mathrm{Glc}(1\right]_{X} \rightarrow 3)\mathrm{Glc}(1 \left[\rightarrow \begin{array}{c}\mathrm{Glc}(1 \rightarrow 6)\mathrm{Glc}\\ 1\\ \downarrow\\ 6\\ 3)\mathrm{Glc}(1\end{array}\right]_{Y} \rightarrow \right]_{m}$$

and formula II

$$\left[ \left[\rightarrow 3)\mathrm{Glc}(1\right]_{X'} \rightarrow \begin{array}{c}\mathrm{Glc}(1 \rightarrow 4)\mathrm{Glc}\\ 1\\ \downarrow\\ 6\\ 3)\mathrm{Glc}(1\end{array} \left[\rightarrow 3)\mathrm{Glc}(1\right]_{Y'} \rightarrow 6)\mathrm{Glc}(1 \rightarrow \right]_{n}$$

wherein each of X, Y, X' and Y' independently represents 1 or an integer greater than 1, each of X+Y and X'+Y' independently represents an integer in the range of from 3 to 5, and the ratio m:n is from 1:3 to 1:5.

6. A process for preparing a beta-D-glucan as claimed in Claim 1, comprising:

culturing a microorganism of the genus <u>Aureobasidium</u>

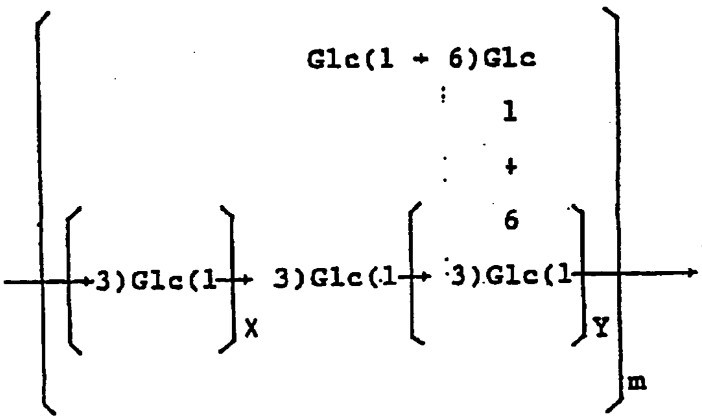
capable of producing said beta-D-glucan to accumulate the beta-D-glucan in the culture; and recovering the accumulated beta-D-glucan.

7. A process according to Claim 6, wherein said beta-D-glucan, upon methylation analysis, gives molecular ratios of 11 to 15 for 2,4,6-tri-O-methyl-D-glucose; 0.8 to 1.2 for 2,3,4-tri-O-methyl-D-glucose; 0.6 to 1.0 for 2,3,6-tri-O-methyl-D-glucose; and 0.8 to 1.2 for 2,4-di-O-methyl-D-glucose against 1.0 mole of 2,3,4,6-tetra-O-methyl-D-glucose.

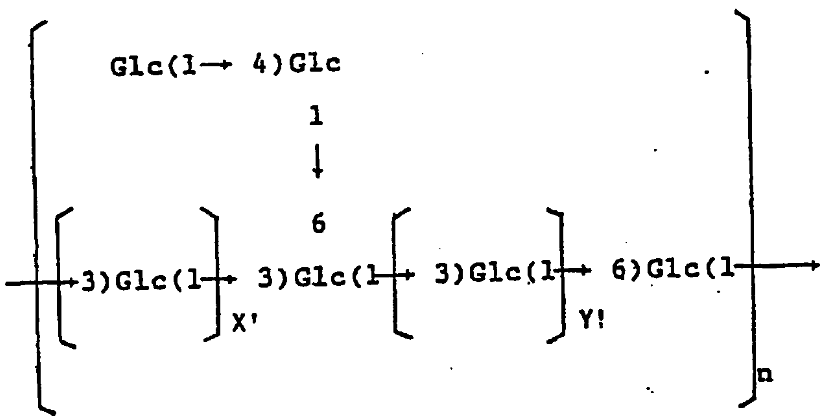
8. A process according to Claim 6, wherein said beta-D-glucan, upon methylation analysis, gives molecular ratios of 3 to 5 for 2,4,6-tri-O-methyl-D-glucose; 0.3 to 0.5 for 2,3,4-tri-O-methyl-D-glucose; 0.2 to 0.4 for 2,3,6-tri-O-methyl-D-glucose; and 0.9 to 1.2 for 2,4-di-O-methyl-D-glucose, against 1.0 mole of 2,3,4,6-tetra-O-methyl-D-glucose.

9. A process according to Claim 7, wherein said beta-D-glucan is mainly composed of repeating units as represented by the following formulae:

formula I



and formula II



wherein each of X, Y, X' and Y' independently represents 1 or an integer greater than 1, each of X+Y and X'+Y' independently represents an integer in the range of from 11 to 15, and the ratio m:n is from 1:3 to 1:5.

10. A process according to Claim 8, wherein said beta-D-glucan is mainly composed of repeating units as represented by the following formulae:

formula I

$$\left[ \left[ \to 3)\mathrm{Glc}(1 \right]_{X} \to 3)\mathrm{Glc}(1 \left[ \to \begin{array}{c} \mathrm{Glc}(1 \to 6)\mathrm{Glc} \\ 1 \\ \downarrow \\ 6 \\ 3)\mathrm{Glc}(1 \end{array} \right]_{Y} \to \right]_{m}$$

and formula II

$$\left[ \left[ \to 3)\mathrm{Glc}(1 \right]_{X'} \to \begin{array}{c} \mathrm{Glc}(1 \to 4)\mathrm{Glc} \\ 1 \\ \downarrow \\ 6 \\ 3)\mathrm{Glc}(1 \end{array} \left[ \to 3)\mathrm{Glc}(1 \right]_{Y'} \to 6)\mathrm{Glc}(1 \to \right]_{n}$$

wherein each of X, Y, X' and Y' independently represents 1 or an integer greater than 1, each of X+Y and X'+Y' independently represents an integer in the range of from 3 to 5, and the ratio m:n is from 1:3 to 1:5.

11. A process according to any one of Claims 6 to 10, wherein the recovery step comprises:

extracting the beta-D-glucan from the cells or cell-

walls with an alkaline solution; and

recovering the beta-D-glucan.

12. A process according to any one of Claims 6 to 11, wherein pullulan is simultaneously produced.

13. A composition containing at least one beta-D-glucan as claimed in any one of Claims 1 to 5, and/or at least one polyol derivative thereof.

14. A composition according to Claim 13, wherein the polyol derivative has been obtained by the steps of oxidizing the beta-D-glucan with periodic acid and/or a water soluble salt thereof; and reducing the oxidized beta-D-glucan.

15. A composition according to Claim 13 or 14, which has been formed into a shaped product by moulding.

16. A composition according to Claim 13 or 14, which is a food product.

17. A composition according to Claim 13 or 14, which is an antioncotic agent.

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FIG. 1